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# Enzyme replacement therapy in Mucopolysaccharidosis VI: evidence for immune responses and altered efficacy of treatment in animal models

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**Abstract**

Enzyme replacement therapy (ERT) can potentially result in an immunological response to the introduced protein. The immunological response by Mucopolysaccharidosis type VI (MPS VI) cats to recombinant human *N*-acetylgalactosamine 4-sulfatase (rh4S) ERT has been investigated. Plasma antibody titres to rh4S were detected in untreated MPS VI and normal control cats, but the antibody titres to rh4S were higher in ERT treated MPS VI cats. The reactivity by cats to rh4S did not appear to be just due to species cross reactivity, as plasma antibodies from normal control, MPS VI and MPS VI ERT cats reacted equally with feline and human 4-sulfatase. Normal control and MPS VI human plasma also had antibody titres to rh4S. Plasma antibodies to rh4S, from an ERT treated cat, could be temporarily removed from circulation by enzyme infusion, confirming specificity for rh4S and indicating a possible window for ERT in the absence of antibody. In enzyme distribution studies with $^{3}$H-rh4S, evidence of altered targeting, and enzyme inactivation and degradation were observed in high compared to low titre rats. In high titre rats, the observed loss of $^{3}$H-label from vacuolar organelles of the liver may represent either degradation of antibody bound $^{3}$H-rh4S for reutilisation within the liver, or antigen presentation. The development of high titre antibody may have a detrimental effect on the efficacy of ERT. © 1997 Elsevier Science B.V.

*Keywords:* Lysosomal storage disease; Mucopolysaccharidosis VI; Treatment outcome; Immune reaction; Antibody

## 1. Introduction

Mucopolysaccharidosis type VI (MPS VI)[1], or Maroteaux-Lamy syndrome (McKusick 25330), is an autosomal recessively inherited disorder which results from a deficiency in the lysosomal hydrolase *N*-acetylgalactosamine 4-sulfatase (4S, EC 3.1.6.1). The archetype for MPS VI clinical presentation includes short stature, kyphosis, coarse facies, dysostosis multiplex, joint stiffness, heart valve problems, hepatosplenomegaly and corneal clouding. The onset of clinical signs in this severe form of the disorder is rapid (at age $\leq 2$ yr) and there is severe progression until the patient dies, usually in their early teenage years [1,2].

The isolation and characterisation of the cDNA sequence for human 4S (h4S: [3,4]) has permitted the

Abbreviations: CHO, Chinese hamster ovary; ERT, enzyme replacement therapy; MPS VI, Mucopolysaccharidosis type VI; 4S, N-acetylgalactosamine 4-sulfatase; rh4S, recombinant human 4-sulfatase; $^{3}$H-rh4S, radiolabeled recombinant human 4-sulfatase h4S, human 4-sulfatase; f4S, feline 4-sulfatase; rf4S, recombinant feline 4-sulfatase; PBS, phosphate buffered saline; MUS, 4-methylumbelliferyl sulfate; Ig, immunoglobulin; iv, intravenous

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investigation of the molecular genetic defects giving rise to MPS VI. The mutant alleles described thus far indicate that most MPS VI mutations are unique [5], suggesting that there should be a broad molecular and clinical heterogeneity for MPS VI. A common feature of MPS VI, is that all patients have less than 5% 4S catalytic capacity, which is associated with a lowered cellular content of 4S protein [6,7]. As almost all patients have some detectable mutant 4S, it is expected that the patient's immune system will have been exposed to 4S protein, albeit mutant protein. This is an important factor with regard to treatment regimens which intend to replace the 4S enzyme deficiency with a source of functional and therefore potentially immunogenic protein.

To date several treatment regimens have been postulated for patients with MPS and other lysosomal storage disorders [8–13]. One proposed therapy, enzyme replacement therapy (ERT) has been successfully used in correcting MPS VII in a mouse model [11] and clinical trials in humans with Gaucher disease have been established and have yielded promising results [14–16]. These studies have all reported evidence of immune reactivity to the replacement protein in some individuals. The development of antibodies in Gaucher patients receiving human ERT suggests that the normal enzyme is recognized as structurally different from the mutant protein, by the patients immune system. Another study has reported the evaluation of ERT in a canine model of Mucopolysaccharidosis type I and demonstrated antibodies to $\alpha$-L-iduronidase in response to enzyme infusion [12]. Interestingly, the treatment of lysosomal storage disorder patients by bone marrow transplantation may avoid the problems of antibody reactivity to the replacement protein, as the engrafted marrow would have already been exposed to this normal protein.

MPS VI cats have been subjected to ERT with recombinant h4S (rh4S), as a prelude to clinical trials in human MPS VI patients [17,18]. These studies demonstrated reduced lysosomal storage in the liver, skin fibroblasts, aorta, heart valve and in brain perivascular cells of MPS VI cats treated with rh4S, when compared to untreated MPS VI controls [17,18]. Skeletal pathology was also reduced in the MPS VI ERT treated cats [18].

The administration of human enzyme into the cat potentially raises the problem of species cross-reacting antibodies. Until recently, the sequence for feline 4S (f4S: [19]) and therefore the expression systems required to produce large amounts of f4S have not been available. The sequence for f4S does however reveal greater than 90% homology with h4S [19] and both have been shown to have very similar immunochemical properties [20]. A panel of seven different conformation dependent monoclonal antibodies raised against h4S showed only low reactivity with f4S, but when first bound by a polyclonal antibody to h4S, the f4S appeared to be conformationally altered, exposing the epitopes and allowing similar detection of f4S and h4S with the same monoclonal antibodies [20]. This minor conformation difference between f4S and h4S may approximate the reported conformation difference between the mutant h4S in MPS VI patients and the normal rh4S to be infused during ERT.

It is postulated that a number of enzyme treated patients will develop circulating antibodies to the replacement protein and that antibody reactivity may in some cases prove to be detrimental to the efficacy of the treatment. Here we report antibodies in untreated normal control cats and normal control humans which both react with rh4S, and characterize the immune response to rh4S ERT in MPS VI cats. To investigate the effect of developing high titre antibodies in response to ERT, the distribution and fate of radiolabeled rh4S ($^3$H-rh4S) was studied in a model system using high and low titre rats.

## 2. Materials and methods

Polyvinylchloride plates (96 well) and 75 cm$^2$ culture flasks were obtained from Costar (Cambridge, MA, USA). Sheep anti-mouse immunoglobulin and peroxidase labeled sheep anti-mouse immunoglobulin reagents were purchased from Silenus Laboratories (Melbourne, Victoria, Australia). Goat anti-cat immunoglobulin and peroxidase labeled goat anti-cat immunoglobulin were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Rabbit anti-rat immunoglobulin and peroxidase labeled rabbit anti-rat immunoglobulin reagents were purchased from Sigma Immunochemicals, Sigma-Aldrich Pty. (Castle Hill, New South Wales, Australia). Sheep anti-human immunoglobulin and peroxidase

labeled sheep anti-human immunoglobulin were from Silenus Laboratories (Melbourne, Victoria, Australia). ABTS substrate kits and Affi-Gel 10 were from Bio-Rad Laboratories (Richmond, VA, USA) and were used according to the manufacturers' instructions. 4-Methylumbelliferyl sulfate (MUS) was obtained from Koch-Light, Haverhill, Suffolk, UK. PBS without magnesium or calcium was from CSL Limited (Parkville, Victoria, Australia). NCS tissue solubilizer was from Amersham corporation (Arlington Heights, IL, USA). Optiphase 'Hi Safe 3' liquid scintillant and a Wallac 1409 liquid scintillation counter were from Wallac Oy (Turku, Finland).

### 2.1. Expression and purification of rh4S

The CHO-DK1 4S expression cell line was as previously described [21]. rh4S was purified to homogeneity from CHO cell culture medium by immunoaffinity chromatography, as previously described [17]. $^{3}$H-rh4S was produced in the same CHO expression cells and was labeled with $^{3}$H-leucine based on a method previously described for the labeling of 4S in fibroblasts [22]. The ratio of radioactivity (dpm) to 4S activity (nmol/min) for the $^{3}$H-rh4S was either 44 or 76 dpm/nmol per min. The specific enzyme activity of the $^{3}$H-rh4S protein was either 49 000 or 54 000 nmol/min per mg and the purity was assessed as previously described [17], except that the protein was visualized by autoradiography (70% precursor (66 kDa) and 30% mature (43, 8, 7 kDa) 4S polypeptide, with no other polypeptides observed).

### 2.2. Feline model for MPS VI and production of rf4S

A colony of MPS VI cats was established [17], from a colony described by Haskins and colleagues [23–25]. The phenotype exhibited by homozygous affected cats was consistent with a severe form of the disorder and the clinical presentation observed was similar to that of human patients with MPS VI [17]. Homozygous MPS VI affected and heterozygous cats were distinguished on the basis of urinary glycosaminoglycan screening, as developed for the diagnosis of human MPS VI patients [26] and the identification of a specific genotype causing the disease [27]. The rf4S was produced and purified as previously described [27].

### 2.3. Infusion of rh4S and plasma sampling in cats

Injection of rh4S (in PBS at 1–3 mg/ml) by i.v. infusion was as previously described [17], usually at a rate of 5–10 ml/kg per h, but no more than 90 ml/kg per h. One cat was treated from birth by s.c. injection of rh4S. Plasma samples were collected as previously described [17]. Cats suspected of exhibiting immunological reactions were premedicated with Periactin® (Cyproheptadine, orally, 1–2 mg; Charles E. Frosst Australia Pty., South Granville, N.S.W., Australia), Niramine® (Chlorpheniramine maleate, subcutaneously, 1.5–2.5 mg total; Coopers Veterinary Products, Silverwater, N.S.W., Australia; or Piriton® orally at 1–2 mg, Glaxo Australia Pty.), and Solucortef® (Hydrocortisone sodium succinate, intravenously, 5 mg/kg; Upjohn Pty., Rydalmere, N.S.W., Australia), and were infused with rh4S at less than 5 ml/kg per h.

### 2.4. Immunoassay of anti-4S antibodies

An ELISA assay was used to determine the titre of either anti-rh4S or anti-rf4S antibodies, from either human, feline or rat plasma. A 50 μl aliquot of rh4S or rf4S (10 μg/ml in PBS pH 7.2) was added to each well of a polyvinyl chloride plate and incubated for 2 h at 37°C followed by overnight incubation at 4°C. The unbound 4S was aspirated from each well and then any remaining reactive sites on the plate blocked by three washes of 200 μl of 0.02 M Tris/HCl, pH 7.0 containing 0.25 M NaCl and 1% (w/v) ovalbumin. After the third wash the wells were incubated in 200 μl of the same buffer for at least 2 h at 20°C. Test samples and control plasma were pre-absorbed with ovalbumin/BSA coupled Affi-Gel 10 (10 mg of each protein was coupled to 1 ml of Affi-Gel 10, using the manufacturers instructions), then serially diluted in 0.02 M Tris/HCl, pH 7.0 containing 0.25 M NaCl and 1% (w/v) ovalbumin and incubated on the 4S coated wells (4 h at 20°C). Each well was then aspirated and washed three times with 200 μl of 0.02 M Tris/HCl, pH 7.0, containing 0.25 M NaCl and 1% (w/v) ovalbumin. Wells were then aspirated and incubated with 100 μl of either a peroxidase-labeled goat anti-cat Ig or peroxidase-labeled sheep anti-human Ig or peroxidase-labeled rabbit anti-rat Ig (1/2000 dilution (v/v)

in 0.02 M Tris/HCl, pH 7.0 containing 0.25 M NaCl and 1% (w/v) ovalbumin), for 1 h at 20°C. The unbound antibody was aspirated and the wells washed three times in 200 μl 0.02 M Tris/HCl, pH 7.0 containing 0.25 M NaCl, before adding 100 μl per well of a peroxidase substrate solution (ABTS substrate kit). After 20 min incubation at 20°C, colour development was quantified by measuring absorbance at 414 nm on an automated ELISA reader (Ceres 900 HDi, Bio Tek Instruments, Winooski, VT, USA). All anti-rh4S or anti-rf4S reactivities were compared to a negative control which determined the reactivity of plasma with ovalbumin.

### 2.5. Immunoblotting of rh4S with cat and rat sera

Immunoblotting was performed as previously described [6], except that the reactivity of the cat sera with rh4S was detected by a peroxidase-labeled goat anti-cat Ig and the reactivity of the rat sera with rh4S was detected by a peroxidase-labeled rabbit anti-rat Ig.

### 2.6. In vitro enzyme uptake studies

The effect of anti-rh4S antibody on rh4S uptake into feline fibroblasts was investigated, using plasma from a treated MPS VI cat (titre 1 024 000). Feline fibroblasts were grown to confluence in 9.6 $cm^2$ culture wells. A 1:5 dilution of either feline plasma, or fetal bovine serum control was incubated with rh4S (10 or 30 nmol/min/ml in complete cell culture medium), for 5 h at 4°C. 2 ml of the antibody incubated rh4S was then added to fibroblast monolayers and enzyme uptake allowed to proceed for 24 h at 37°C. The medium was then aspirated and the cell layers washed three times with PBS before the preparation and assay of cell extracts as previously described [7].

### 2.7. In vivo enzyme uptake studies in rats

To determine the effect of anti-rh4S antibody on enzyme distribution in vivo, normal control rats were selected either on the basis of low titres to rh4S (titre 64) or were immunized with rh4S to induce antibody responses (titre 1 024 000) and then infused with $^3$H-rh4S. Anti-rh4S antibodies were induced in normal control animals by injection of 50 μg doses of purified rh4S in Freund's adjuvant. One injection of Freund's complete adjuvant (0.15 ml adjuvant with 0.15 ml of rh4S in PBS) was given subcutaneously as a priming dose and followed by three subcutaneous injections of rh4S in Freund's incomplete adjuvant at three weekly intervals. Plasma samples for antibody titre determination were obtained by tail vein bleeding of anaesthetized rats. Antibody titres in rats were confirmed by ELISA of plasma antibodies, then the animals were injected i.v. with 1 mg/kg of $^3$H-rh4S ($1.25–1.8 \times 10^6$ dpm $^3$H-rh4S). Rats were sacrificed at either 30 or 60 min after injection and analysed for the distribution of $^3$H-rh4S in different tissues.

### 2.8. Granular fractionation of rat liver

Five grams of rat liver was placed in 45 ml of 0.25 M sucrose containing 1 mM EDTA, pH 7.5 (4°C) then dissected on ice and passaged through stainless steel mesh. The cell suspension was centrifuged at $100 \times g$ for 5 s to remove aggregates and connective tissue, and the supernatant then recentrifuged at $200 \times g$ for 10 min at 4°C. The resulting pellet was resuspended to 10 ml in 0.25 M sucrose containing 1 mM EDTA, pH 7.5 (4°C) and homogenized in a Potter-Elalijem using 5 strokes. The homogenate was then centrifuged at $200 \times g$ for 10 min to remove the cellular debris and the supernatant passed through a 23 gauge needle to dissociate any adhering organelles. This granular fraction was then separated on either a 10% Percoll gradient (30 min experiment) or 18% Percoll gradient (60 min experiment) and assayed as previously described [28].

### 2.9. Detection of rh4S activity in rat tissue extracts

Rat tissue extracts were prepared as previously described for feline tissues [20]. An enzyme immunobinding assay was used to specifically detect 4S activity. The procedure used was as described previously [29], except that an affinity purified sheep anti-mouse immunoglobulin antibody (1 μg/well in 0.1 M $NaHCO_3$ pH 8.5) was bound to each well of a 96-well polyvinylchloride plate, before the addition of a monoclonal antibody which specifically reacts with h4S (4-S F66 [20] in this case as hybridoma culture supernatant). Antibody bound rh4S was detected with MUS substrate and the results expressed as nmol/min g of tissue wet weight.

### 2.10. Detection of $^{3}$H-rh4S in tissue extracts

To determine the level of $^{3}$H-rh4S in different tissues, samples were dissected into small fragments and solubilized overnight at 22°C in 1 M NaOH (5 ml/g of tissue). 200 μl of sample was neutralized with HCl then mixed with 500 μl of NCS tissue solubilizer/isopropanol (1:2 v/v) for 15 min at 22°C. 100 μl of 30% $H_2O_2$ was then added, mixed and allowed to incubate for 60 min at 22°C. Optiphase 'Hi Safe 3' liquid scintillant (15 ml) was then added and the samples counted on a Wallac liquid scintillation counter. Results were expressed as dpm/g of tissue wet weight.

## 3. Results

### 3.1. Feline and human antibody reactivity to rh4S

Plasma antibody titres to rh4S were detected in both untreated MPS VI (titre 8,390 ± 7,277) and normal control cats (titre 10 343 ± 13 846) (Fig. 1). There was no significant difference between the reactivity of plasma, from either untreated MPS VI ($n$ = 20) or normal control cats ($n$ = 33), with rh4S (Tukey-B test; Fig. 1). There was also no significant difference between the reactivity of plasma from eleven untreated MPS VI and normal control cats, with rf4S when compared to rh4S (Student's $t$-test, $P = 0.421$, data not shown). The mean of plasma titres to rh4S, was higher for rh4S treated MPS VI cats ($n$ = 17, 173 397 ± 351 150, Fig. 1) when compared to either untreated MPS VI or normal control cats. This difference was statistically significant (Tukey-B test, $P = 0.05$). However, 13 of 17 MPS VI cats which were treated with rh4S, had anti-rh4S antibody titres less than 44 000 (the upper limit for normal control cats, Fig. 1), after 3–15 mth of either weekly or bi-weekly rh4S infusions, whereas four rh4S treated MPS VI cats had anti-rh4S antibody titres above the upper limit for normal control cats (Fig. 1). One of the latter four cats was treated from birth by s.c. injection of rh4S, while two others had i.v. ERT initiated at either 2 or 7 mth after birth. Only one of fourteen cats treated from birth by i.v.

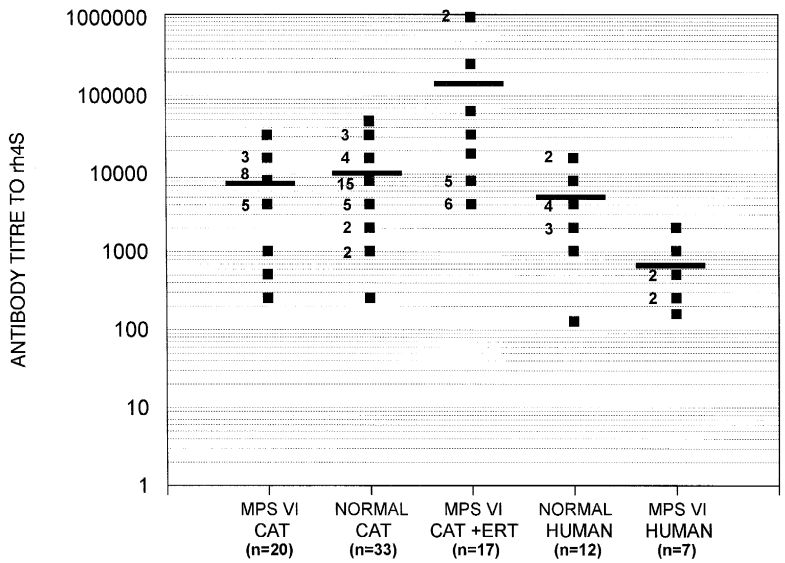


Fig. 1. Antibody titres to rh4S in cats and humans. Untreated MPS VI and normal control, cats and humans were not injected with rh4S. MPS VI treated cats received doses of 0.5–5 mg/kg of rh4S, at either 0.5, 1, or 2 weekly intervals. Plasma samples were isolated and tested as described in Section 2. Results represent the highest anti-rh4S antibody titre recorded from monthly measurements. The numbers on the figure represent the number of samples with that antibody titre. Statistical analysis of the anti-rh4S antibody titres in cats, demonstrated a difference between normal control cats and MPS VI treated cats, and a difference between untreated MPS VI cats and MPS VI treated cats (Tukey-B test, $P = 0.05$). There was no significant difference between normal control cat, untreated MPS VI cat and normal control human plasma antibody titres to rh4S. Untreated human MPS VI patient plasma had significantly lower antibody titres to rh4S compared to both normal control humans and any of the cat groups (Tukey-B test, $P = 0.05$).

infusion had an anti-rh4S titre higher than the range for either untreated MPS VI cats or normal control cats. Taken as a subgroup of rh4S ERT treated cats, the cats treated from birth by iv infusion had anti-rh4S antibody titres which were not significantly different from either untreated MPS VI or normal control cats (Tukey-B test). In experiments involving the MPS VI cats treated with iv rh4S from 2 and 7 mth of age (Fig. 2), a progressive increase in antibody titre was observed during treatment, reaching maximal titres of either 64 000 (cat #84) or 1 024 000 (cat #40). Cats which produced antibody titres to rh4S after ERT had similar titres to both rh4S and rf4S (data not shown).

Antibodies with reactivity to rh4S were also detected in normal control human plasma (Fig. 1: titre $5{,}322 \pm 5{,}392$) and human MPS VI patient plasma (Fig. 1: titre $681 \pm 668$). There was a significant difference (Tukey-B test, $P = 0.05$), between the anti-rh4S antibody titres for untreated human MPS VI patient plasma and either normal control human plasma or any of the cat plasma antibody titres (ie. either normal control, or MPS VI, or MPS VI ERT treated cats). There was no significant difference between the antibody titres to rh4S for either normal control human plasma, or normal control cat plasma or untreated MPS VI cat plasma. Notably, a human MPS VI patient with a truncation mutation which resulted in no detectable 4S protein still had plasma antibody reactivity to rh4S (titre 160).

Immunoblotting to detect rh4S, was performed using feline plasma from rh4S ERT treated MPS VI cats (titre 1 024 000, Fig. 3a) and untreated MPS VI cats (titres 16 000 and 2,048). Immunoblot reactivity to rh4S was only detected with the antibodies from the MPS VI cats treated with rh4S (Fig. 3a), with no detectable reactivity to rh4S observed for the untreated MPS VI cats (data not shown). Immunoblot reactivity to rh4S was also observed for plasma from high titre (immunized) rats, but little or no reactivity was observed with low titre non-immune rat plasma.

### *3.2. Clinical / immunological response of cats to infused rh4S*

All cats demonstrated to a lesser or greater extent signs of adverse responses to infused rh4S at some point during ERT treatment. Responses did not appear to correlate with the level of antibody titre but tended to be more obvious in cats treated with 5 mg/kg of rh4S. Symptoms of responding cats included trembling, ear scratching, increased respiratory rate, pyrexia, anxiety, cyanosis, vomiting, diarrhoea, and respiratory distress. In most cases responses were limited to ear scratching and vomiting,

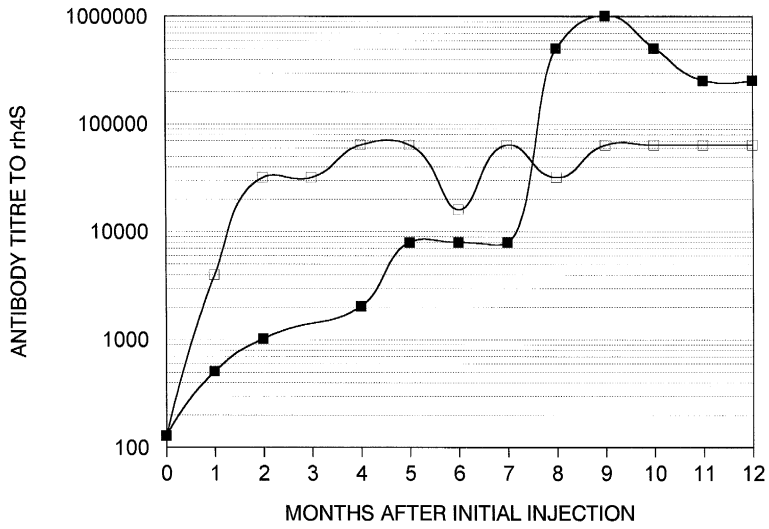


Fig. 2. Development of antibody titres to rh4S, in rh4S infused MPS VI cats. Antibody titres to rh4S were determined on plasma samples from two MPS VI cats following the initiation of long term rh4S ERT. Titres were monitored at monthly intervals. Cat # 40 (– ■ –) and cat #84 (– □ –) received rh4S iv at 1 mg/kg each fortnight and treatment was initiated at either 7 mth or 2 mth of age, respectively. Plasma samples were prepared and assayed by ELISA as described in Section 2.

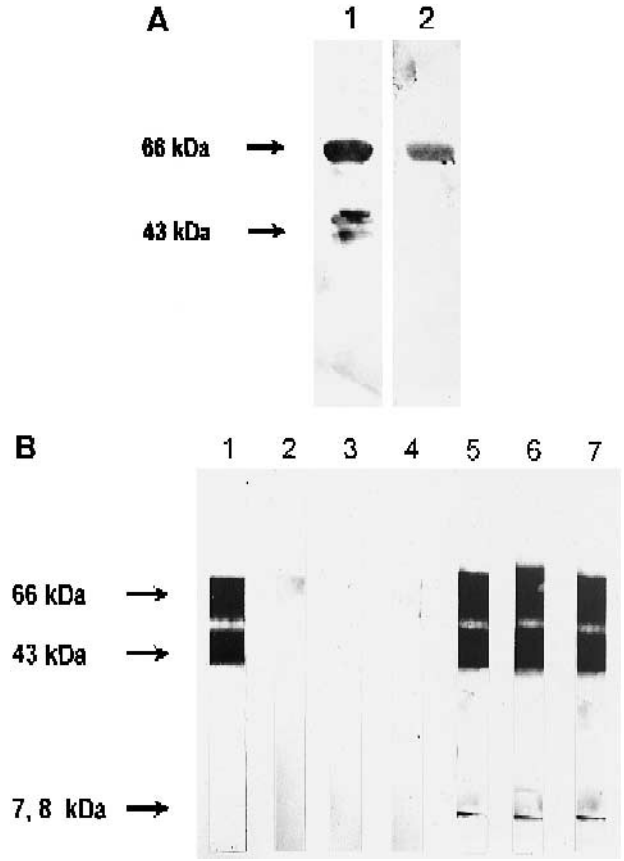


Fig. 3. Immunoblot analysis of anti-rh4S antibodies from MPS VI ERT treated cats (a) and high and low titre rats (b). 5 $\mu$g of rh4S was electrophoresed per lane by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting. a. Immunoblots from two MPS VI rh4S treated cats (titres 1 024 000, lanes 1–2), while two untreated MPS VI cats did not react (titre 16 000 and 2,048: data not shown). b. A 4-sulfatase protein stain (lane 1), and immunoblots from three low titre rats (lanes 2–4) and three high titre rats (lanes 5–7).

with only a few cats showing severe reactions involving all of the described symptoms. Responding cats were pre-treated with Periactin®, Niramine®/Piriton®, and Solucortef® as described in Materials and Methods and were infused with rh4S at no more than 5 ml/kg per h, which appeared to reduce responsiveness. In cases where reactivity was suspected, infusion was stopped and resumed 60 min later.

### *3.3. Effect of anti-rh4S antibody on rh4S uptake in fibroblasts*

rh4S uptake experiments were conducted in feline fibroblasts, in the presence or absence of anti-rh4S antibody from an rh4S treated MPS VI cat (cat #40, titre 1 024 000; Table 1). The incubation of rh4S with feline fibroblasts resulted in high levels of 4S uptake over 3 days. The addition of plasma antibody to rh4S prior to uptake, reduced the levels of active rh4S detected in cell extracts. Increasing the concentration of rh4S relative to the concentration of antibody added, partially overcame the observed affect on uptake (Table 1).

### *3.4. Modification of antibody titre to rh4S after in vivo injection of rh4S protein*

The level of anti-rh4S antibody was monitored in an MPS VI cat treated with rh4S (cat #40, titre 1 024 000), following the iv infusion of a 1 mg/kg dose of rh4S (Fig. 4). The plasma sample taken immediately prior to the injection of the rh4S, had an antibody titre of 1 024 000. The volume of the rh4S

Table 1
Antibody inhibition of the uptake of active rh4S into feline fibroblasts

| Conditions | 4S activity (nmol/min per mg) | | | |
|---|---|---|---|---|
| | 10 nmol/min per ml rh4S no antibody | 10 nmol/min per ml rh4s + antibody | 30 nmol/min per ml rh4S no antibody | 30 nmol/min per ml rh4s + antibody |
| Time (h) | | | | |
| 0 | 32 | 32 | 32 | 32 |
| 24 | 168 | 47 | 149 | 78 |
| 42 | 227 | 43 | 336 | 162 |
| 68 | 426 | 74 | 438 | 333 |

Feline fibroblasts were cultured in 25 $cm^2$ culture dishes as previously described [6]. Fibroblasts were incubated for the specified times in the presence of either 10 or 30 nmol/min per ml (in 5 ml total) of rh4S, in basal modified eagle's medium containing 10% heat inactivated fetal calf serum. + Antibody represents cultures treated with plasma antibodies from a rh4S treated cat (cat #40), where the final dilution of feline plasma was 1:20 (i.e. titre of 50 000 from original titre of 1 024 000).

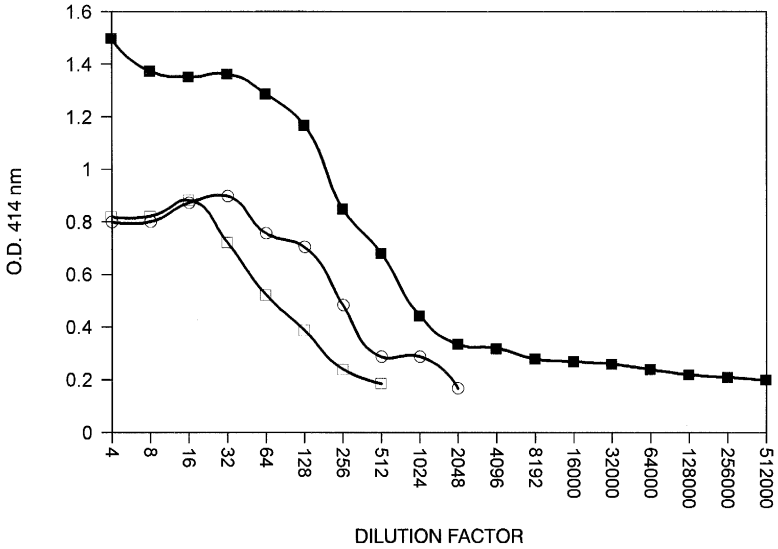


Fig. 4. In vivo depletion of rh4S reactive antibodies from circulation. rh4S was injected into cat # 40 (1 mg/kg, iv) and the effect on the anti-rh4S antibody titre monitored at time zero (– ■ –), 2 min (– ∘ –) and 60 min (– □ –) after injection. Plasma samples were isolated and tested by ELISA as described in Section 2. Results are expressed as absorbance at 414 nm.

Table 2
Distribution of $^{3}$H-rh4S in a low titre rat and a high titre rat, 30 min after infusion

| Organ | Low titre | | | High titre | | |
|---|---|---|---|---|---|---|
| | (dpm/g) | (nmol/min per g) | (dpm/nmol per min) ** | (dpm/g) | (nmol/min per/g) | (dpm/nmol per min) ** |
| liver | 77 968 | 471 | 166 | 59 700 | 4.8 | 12 438 |
| spleen | 42 271 | 250 | 169 | 30 567 | 2.4 | 12 736 |
| kidney | 2,105 | 18 | 117 | 466 | 0.3 | 1,553 |
| lung | 1,125 | 1.6 | 703 | 22 127 | 1.3 | 17 020 |
| heart | 475 | 3 | 158 | 962 | 0.3 | 3,207 |
| lymph node | 868 | 3.5 | 248 | 2,375 | 0.4 | 5,938 |

Rats (270–280 g), received $1.25 \times 10^{6}$ dpm $^{3}$H-rh4S (76 dpm/nmol per min; 49 000 nmol/min per mg) as a single i.v. injection and were sacrificed after 30 min. The low titre rat (titre 64) received no previous injections of rh4S. The high titre rat (titre 1 024 000) was immunized with rh4S, given as four 50 $\mu$g injections at 2–3 wk intervals. Results are expressed as dpm/gm for $^{3}$H-rh4S radioactivity or as nmol/min per g for 4S activity. **, represents the ratio of dpm/gm to nmol/min per g and is expressed as dpm/nmol per min.

Table 3
Distribution of $^{3}$H-rh4S in low and high titre rats, 60 min after infusion

| Organ | Low titre | | | High titre | | |
|---|---|---|---|---|---|---|
| | (dpm/g) | (nmol/min per g) | (dpm/nmol per min) ** | (dpm/g) | (nmol/min per g) | (dpm/nmol per min) ** |
| liver | 59,020 ± 2,216 | 279 ± 17 | 211 | 51 561 ± 8,136 | 17 ± 4.9 | 3,033 |
| spleen | 26,413 ± 5,233 | 124 ± 43 | 213 | 20 368 ± 3,481 | 4 ± 1.7 | 5,092 |
| kidney | 1,947 ± 888 | 5 ± 1.5 | 389 | 2,460 ± 1,768 | 0.8 ± 0.1 | 3,075 |
| lung | 2,893 ± 587 | 8 ± 8.7 | 361 | 27 664 ± 13 738 | 8 ± 3.6 | 3,458 |
| heart | 1,932 ± 267 | 2.5 ± 0.8 | 772 | 2,652 ± 1,407 | 0.6 ± 0.2 | 4,420 |
| lymph node | 3,405 ± 3,844 | 3.7 ± 1.2 | 920 | 2,526 ± 1,820 | 1 ± 0.1 | 2,526 |

Rats (240–260 g), received $1.8 \times 10^{6}$ dpm $^{3}$H-rh4S (44 dpm/nmol per min; 54 000 nmol/min per mg) as a single i.v. injection and were sacrificed after 60 min. The low titre rats ($n = 3$, titre 64) received no previous injections of rh4S. The high titre rats ($n = 3$, titre 1 024 000) were immunized with rh4S, given as four 50 $\mu$g injections at 2–3 wk intervals. Results are expressed as dpm/g ± SD for $^{3}$H-rh4S radioactivity or as nmol/min per g ± SD for 4S activity. **, represents the ratio of dpm/g to nmol/min per g and is expressed as dpm/nmol per min.

infusion was less than 1% of the cats total blood volume. Two min after the rh4S infusion, a plasma sample showed an antibody titre of 2,048, suggesting the removal of most of the anti-rh4S antibody from circulation. The antibody titre remained at this level or lower for at least 1 h. This experiment was repeated in another MPS VI cat treated with rh4S (titre 8,092), where rh4S infusion resulted in a titre of 4,096 at 2 min after infusion and a titre of 2,048 at 60 min after rh4S infusion.

### *3.5. The distribution of infused $^{3}$H-rh4S in low and high titre rats*

To determine the distribution of rh4S in low titre (titre 64) and high titre rats (titre 1 024 000), animals were infused with $^{3}$H-rh4S and sacrificed at either 30 min or 60 min after enzyme infusion.

Thirty min after the infusion of $^{3}$H-rh4S, high levels of radiolabel and rh4S activity were detected in the liver and spleen of a low titre rat (Table 2). Lower but detectable levels of active $^{3}$H-rh4S were also detected in kidney, lung, heart and lymph node. In a high compared to a low titre rat, similar but slightly reduced levels of $^{3}$H-rh4S radiolabel were detected in liver, spleen, and kidney, but a dramatic increase in radiolabel was observed in the lung (Table 2). In addition, the level of rh4S activity in all tissues from the high titre rat were reduced compared to the level detected in the same tissues from the low titre rat. This was reflected as a higher ratio of radiolabel to rh4S activity in tissues from the high compared to the low titre rat (Table 2).

Sixty min after the infusion of $^{3}$H-rh4S a very similar pattern of $^{3}$H-rh4S radiolabel and activity was observed (Table 3), when compared to that observed for the 30 minute experiment (Table 2). Thus, high titre rats had increased levels of radiolabel in the lung compared to low titre rats and all of the high titre rat tissues had low levels of rh4S activity compared to the amount of radiolabel detected (Table 3). The major difference between the 30 and 60 min experiments, was that the ratios of radioactivity to rh4S activity in liver, spleen and lung from the 30 min high titre rat (Table 2), were high compared to the other tissues in the same rat and high compared to the same tissues in the 60 min high titre rats (Table 3).

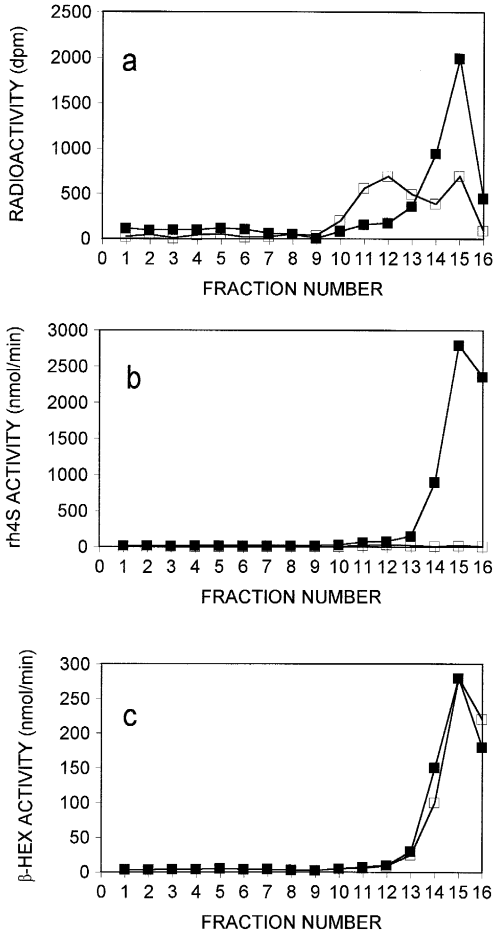


Fig. 5. Granular fractionation (11% Percoll gradient) of rat liver from a high titre rat and a low titre rat, 30 min after the infusion of $^{3}$H-rh4S. a. Distribution of $^{3}$H-rh4S radiolabel in the low titre rat (– ■ –) and the high titre rat (– □ –), and results are expressed as dpm per 1 ml fraction from the Percoll gradient. b. Distribution of rh4S activity in the low titre rat (– ■ –) and the high titre rat (– □ –). c. Distribution of $\beta$-hexosaminidase activity in the same two gradients.

### *3.6. Granular fractionation of rat liver from high and low titre rats infused with $^{3}$H-rh4S*

To determine the intracellular fate of rh4S in the presence and absence of anti-rh4S antibody, high and low titre rats were infused with $^{3}$H-rh4S and the liver isolated and prepared for granular fractionation.

In the rat sacrificed 30 min after the infusion of $^{3}$H-rh4S, Percoll separation of rat liver granular fraction demonstrated a different intracellular distribution in the high compared to the low titre rat (Fig. 5). For the low titre rat, the $^{3}$H-rh4S radiolabel was detected

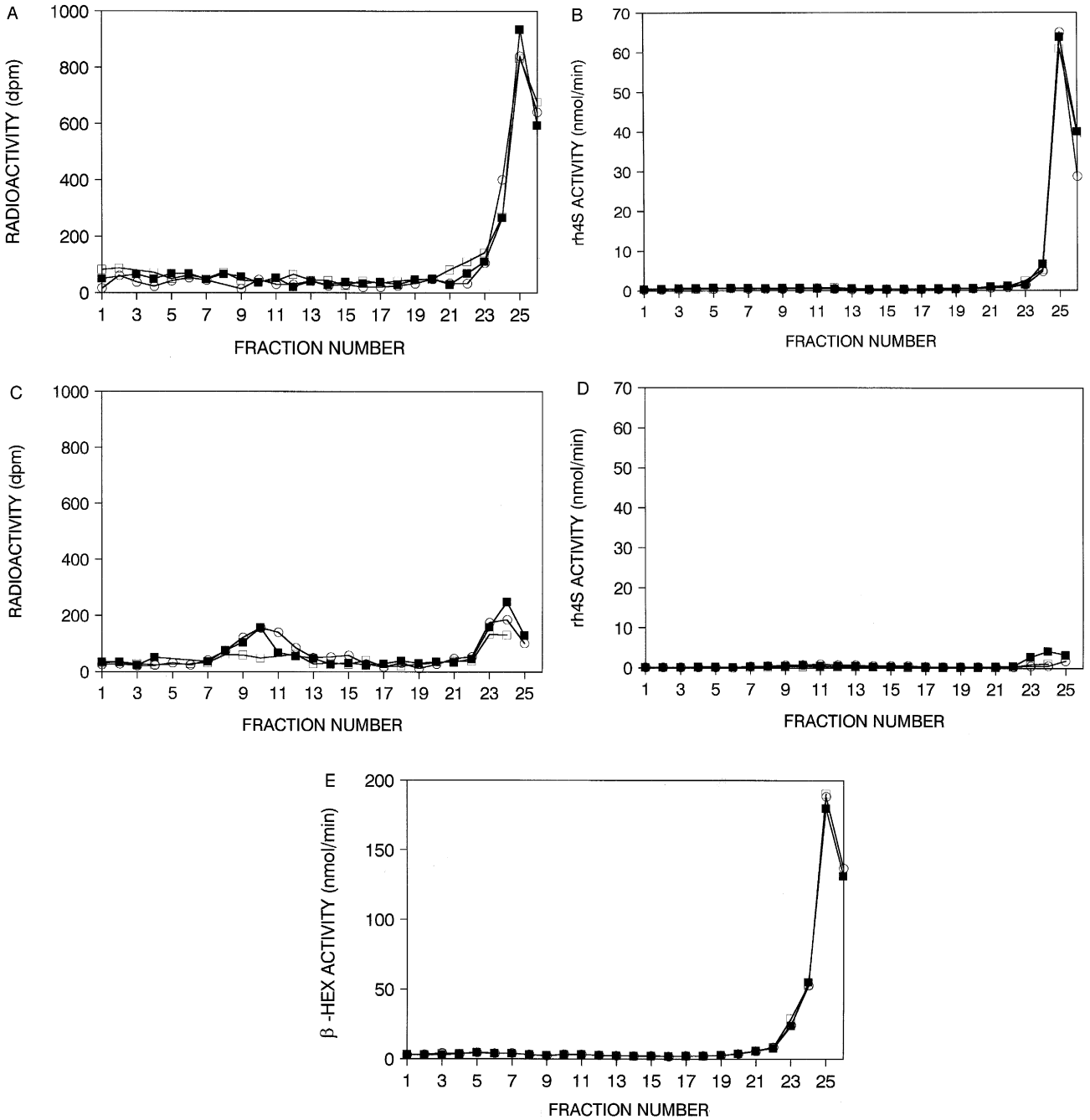


Fig. 6. Granular fractionation (18% Percoll gradient) of rat liver from three high titre rats and three low titre rats, 60 min after the infusion of $^{3}$H-rh4S. Individual rats are represented by either – ■ –, – ∘ –, or – □ –. a. Distribution of $^{3}$H-rh4S radiolabel in Percoll granular fractionation's from three separate low titre rats. b. Distribution of rh4S (measured by an immunobinding assay, [29]) activity in Percoll granular fractionation's from three separate low titre rats. c. Distribution of $^{3}$H-rh4S radiolabel in Percoll granular fractionation's from three separate high titre rats. d. Distribution of rh4S activity (measured by an immunobinding assay, [29]) in Percoll granular fractionation's from three separate high titre rats. In all six granular fractionation's of liver from the low and high titre rats there was an almost identical pattern for the distribution of $\beta$-hexosaminidase activity, and is represented in (e) which shows the distribution in the three high titre rats.

in the high density fractions of the gradient (Fig. 5a, fractions 13–16) which are characteristic of lysosomes and also colocalized with β-hexosaminidase activity (Fig. 5c). While the high titre rat had a similar level of radiolabel in the gradient, a major peak was observed in lower density fractions (Fig. 5a, fractions 10–13), with only some of the radiolabel detected in fractions coincident with β-hexosaminidase activity (Fractions 13–16). The rh4S activity colocalized with the radiolabel for the low titre rat but only very low levels of rh4S activity were detected in the Percoll gradient for the high titre rat, indicating the enzyme had been inactivated (Fig. 5b).

In the low titre rats sacrificed 60 min after the infusion of $^{3}$H-rh4S, the $^{3}$H-rh4S radiolabel (Fig. 6a) and enzyme activity (Fig. 6b) fractionated consistently in high density organelles characteristic of lysosomes. The $^{3}$H-rh4S was also coincident with β-hexosaminidase activity (Fig. 6e). In contrast, the high titre rats showed reduced levels of radiolabel in the gradients (Fig. 6c) and evidence of radiolabel in lower density organelles (Fig. 6c, fractions 7–13). Moreover, there was almost no detectable rh4S activity in the Percoll gradient fractions from these high titre rats (Fig. 6d).

## 4. Discussion

ERT can potentially result in a patient generating an antibody response to the replacement protein, particularly after successive enzyme infusion treatments. The immediate concern for this immunoreactivity is the development of an anaphylactic reaction, which can be life threatening to the patient. Strategies to avert this type of clinical response in patients have been suggested and include slow infusion of enzyme and coadministration of anti-inflammatory drugs. Other possible problems due to the generation of immunoreactivity may involve either antibody inhibition of the replacement enzyme, or inappropriate targeting, or enzyme degradation. These are important concerns for the efficacy of ERT as a proposed treatment for MPS patients.

As a prelude to ERT trials in human MPS VI patients, we have investigated the levels of anti-rh4S antibodies in plasma from normal control humans and human MPS VI patients. Antibody titres to rh4S were observed in both normal control human plasma and untreated MPS VI patient plasma. It could be speculated that the antibody reactivity to rh4S is due to either the exposure of 4S protein to the immune system or reflects a cross reactivity with another antigen. The antibody titre to rh4S detected in an MPS VI patient plasma which had no detectable 4S protein (truncation mutation) indicates that the anti-4S antibodies are probably not produced in response to endogenous 4S protein, but more likely represent a background level of anti-rh4S antibodies produced in response to a cross reacting antigen. The anti-rh4S titre levels were, however, higher in the normal control human plasma when compared to the human MPS VI patient plasma. This may either reflect the small number of samples tested in the human MPS VI patient plasma or that the background level of anti-rh4S antibodies can be increased by exposure to endogenous 4S protein.

The feline model for MPS VI accurately reflects the human disorder, with a very low level of f4S protein [27] and activity giving rise to a severe clinical presentation [17,18,23–25]. Untreated MPS VI and normal control cats were investigated for the level of antibody to rh4S in plasma samples and the development of antibody responses to ERT were monitored in MPS VI cats treated with rh4S. Both untreated MPS VI and normal control cats had antibody titres to rh4S and these antibodies reacted equally well with rf4S, suggesting that the antibodies are not just recognising species specific differences between f4S and h4S. Together with the human plasma data, this suggested that antibodies to 4S are normally found in circulation, an observation which may be important when considering the infusion of 4S protein for ERT.

We have previously reported immune responses in some MPS VI cats undergoing ERT with rh4S [17] and similar responses to human α-L-iduronidase have been reported in an MPS I dog [12]. While it may be argued that species specific antibodies may be generated by injecting rh4S into cats, it is postulated that with the exception of linear sequence differences, the minor structural difference between h4S and f4S [19] will approximate the conformational difference observed between normal rh4S and mutant h4S protein in MPS VI patients [7]. In addition, as discussed above, MPS VI and normal control cat plasma had

similar titres to both rh4S and rf4S. Moreover, evidence from Gaucher studies indicate that human Ceredase® administration into human patients still results in antibody responses in some cases [15,16].

The MPS VI cats treated with rh4S had antibody reactivities to rh4S which were significantly higher to that observed in untreated MPS VI and normal control cats. Plasma antibodies from MPS VI cats treated by rh4S ERT reacted equally with rh4S and rf4S suggesting that this reactivity was not due to a species specific response (data not shown). However, not all rh4S treated MPS VI cats responded with higher than normal levels of anti-rh4S antibody production (reflected by the high standard deviation in plasma titres for the MPS VI ERT treated cats). Three of the four cats responding to rh4S, with titres greater than that observed in untreated MPS VI and normal control cats, were either treated by subcutaneous infusion of rh4S ($n = 1$) or were treated after birth ($n = 2$). For MPS VI cats treated at birth by iv infusion ($n = 14$) only one cat responded with a titre greater than that observed in untreated MPS VI and normal control cats. Taken as a separate treatment group the MPS VI cats treated from birth by iv infusion did not have significantly different anti-rh4S titres compared to the latter two control groups. Treating cats at birth may be advantageous due to the potential to induce tolerance, but a larger sample size, particularly in the post-birth treatment group, is required to confirm this hypothesis. Considering the levels of anti-rh4S antibody titres in untreated MPS VI and normal control cats, the number of cats responding to rh4S infusion with titres higher than the normal range were surprisingly few. Therefore, the background level of anti-rh4S antibodies, observed in untreated MPS VI and normal control cats, did not appear to predispose the ERT treated MPS VI cats to respond to rh4S infusion with higher than normal levels of anti-rh4S antibodies. However, it is expected that some MPS VI cats and human MPS VI patients will respond to ERT with higher than normal levels of anti-rh4S antibodies.

Most rh4S treated MPS VI cats showed at least minor adverse clinical signs of hypersensitivity to rh4S infusion at some stage during the course of treatments, with a trend for cats treated with 5 mg/kg of rh4S to show more reactive signs. The degree of hypersensitivity did not appear to correlate with the antibody titre level, suggesting that responsiveness may be dependent on other parameters such as either antibody isotype or antibody affinity, rather than the amount of circulating antibody. In Gaucher patients, hyperreactivity has been shown to be associated with IgG antibodies rather than IgE antibodies and was mediated by IgG immune complex formation and complement fixation [16,30]. We have been unable to confirm the isotype of the feline immunoglobulin reacting with rh4S, as anti-cat subclass antibodies were not available at the time of this study, but we did show that plasma from high titre cats reacted on immunoblots, suggesting the production of high affinity antibody. The reactivity of high affinity antibodies with rh4S by immunoblotting, was confirmed for plasma antibodies from immunized rats,

Circulating antibodies to 4S could be detrimental to successful ERT, by either inhibition of rh4S activity, or by mediating the premature degradation of rh4S, or by affecting the targeting of the enzyme. In a previous study 4S activity was inhibited by plasma antibodies produced in a rh4S treated MPS VI cat [17]. Moreover, in enzyme uptake studies in feline fibroblasts, the presence of antibody reduced the detectable level of 4S in cell extracts. This reduced uptake of active enzyme, could be due to either direct inhibition of 4S activity, the effect of antibody on the uptake process, or antibody induced degradation, or a combination of mechanisms. In in vivo experiments using cats with anti-rh4S antibody (titres 1 024 000 and 8,092), anti-4S antibodies could be temporarily removed from circulation by rh4S infusion, confirming the specificity of these antibodies for rh4S. This also suggests that with slow infusion and appropriate pre-medication to avoid hypersensitivity reactions, that antibodies inducing either inappropriate targeting, or rh4S degradation, or rh4S inhibition could be temporarily removed from circulation before the administration of an effective therapeutic dose of enzyme.

Having demonstrated anti-4S antibodies in normal control humans and cats, untreated MPS VI humans and cats, and higher than normal anti-rh4S antibody titres in some ERT treated MPS VI cats, a comparative distribution study was performed in high and low titre rats, to define the effects of circulating high titre anti-rh4S antibodies on the targeting and fate of infused $^{3}$H-rh4S. In high compared to low titre rats

there was a difference in tissue distribution with high titre antibody appearing to cause an increase in the amount of $^{3}$H-rh4S trafficking to the lung. The deposition of immune complexes in the lung may account for some of the anaphylactic type reactions observed in rh4S treated MPS VI cats. High titre antibody also caused a marked reduction in enzyme activity of the $^{3}$H-rh4S in all of the tissues tested, indicating that this level of antibody production may have a direct effect on the efficacy of treatment.

The internalization of similar levels of $^{3}$H-rh4S radiolabel into granular fractions from both high and low titre rats, at 30 min after enzyme infusion, suggests that the antibody did not inhibit rh4S uptake. Rapid distribution of infused rh4S to the lysosomal compartment was observed in low titre rats indicating that receptor mediated uptake and targeting/trafficking to the lysosome is an extremely rapid process. This is consistent with our previous observations which showed a very short half life for the clearance, in cats, of infused rh4S from the circulation [17]. In contrast to the low titre rats, much of the $^{3}$H-rh4S did not appear to reach the lysosomal compartment in high titre rats, presumably due to the attached antibody.

While the intracellular targeting and trafficking of $^{3}$H-rh4S was different in high titre compared to low titre animals, the associated effect on enzyme activity was striking. The rh4S was rapidly inactivated throughout the Percoll gradient, even for the radiolabeled material which had apparently reached the lysosomal compartment in the 30 minute experiment. The reduction of radiolabel in the Percoll gradients from high compared to low titre rats at 60 min after enzyme infusion, suggested that the rh4S was either degraded or removed from the vacuolar compartment. Whatever the mechanism for removal of the $^{3}$H-rh4S from the vacuolar compartment, the process appeared to be rapid, with high titre rats at 60 min having markedly reduced levels of the radiolabel in the organelle fractions, when compared to both 30 and 60 min low titre rats and the 30 min high titre rat.

The generation of antibodies in MPS patients, in response to ERT, would appear to have very important implications for the efficacy of the treatment. It is expected that even low levels of antibody may result in some of the replacement protein failing to reach the appropriate site in an active and stable form. The current findings imply that further strategies may be required to avert the production of specific high titre antibodies in order to avoid the potential detrimental effects on treatment efficacy. Possible strategies to avoid the generation of antibodies to a replacement protein include induction of tolerance, immune suppression, coating the enzyme with polyethylene glycol, increased sialylation of the protein, and liposome encapsulation.

The current study indicates that antibody can alter the tissue distribution of infused rh4S enzyme and can inactivate and result in the rapid degradation of internalized rh4S. Moreover the intracellular distribution of rh4S was different in high and low titre rats suggesting that the antibody bound rh4S may be directed to an intracellular organelle which is distinct from the lysosomal compartment. The site of 4S degradation could be the recently identified MIIC compartment that contains class II antigen, and which has been implicated in antigen processing [31,32]. It would then be speculated that antibody attachment to rh4S is responsible for altered intracellular targeting to this compartment, as well as for initiating the ensuing degradation process. The current study has demonstrated that antibody development may have significant implications toward the efficacy of ERT in MPS VI patients.

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